Pseudomonas cuatrociénegasensis sp. nov., isolated from an evaporating lagoon in the Cuatro Ciénegas valley in Coahuila, Mexico

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Nine Gram-negative, rod-shaped, non-spore-forming isolates with identical or very similar repetitive-sequence-based PCR profiles were recovered from an evaporative lagoon in Mexico. Two strains, designated 1N^T and 3N, had virtually identical 16S rRNA gene sequences and, on the basis of these sequences, were identified as members of the genus *Pseudomonas*, with *Pseudomonas peli* R-20805^T as the closest relative. All nine isolates had practically identical whole-cell protein profiles. The major fatty acids [C_{16:0}, C_{18:1}ω7c and summed feature a (C_{16:1}ω7 and/or C_{16:1}ω6c)] of strains 1N^T and 3N supported their affiliation with the genus *Pseudomonas*. The DNA–DNA reassociation values with respect to *P. peli* LMG 23201^T and other closely related *Pseudomonas* species were <15%. Physiological and biochemical tests allowed phenotypic differentiation of the strains analysed, including strain 1N^T, from the five phylogenetically closest *Pseudomonas* species. On the basis of the data obtained by using this polyphasic taxonomic approach, the nine strains represent a novel species, for which the name *Pseudomonas cuatrociénegasensis* sp. nov. is proposed. The type strain is 1N^T (=LMG 24676^T=CIP 109853^T).

The genus *Pseudomonas* was described by Migula (1894). Since then, there has been a steady increase in the rate of description of species belonging to the genus *Pseudomonas*; there are currently more than 191 species with validly published names (Anzai *et al.*, 2000; http://www.dsmz.de/microorganisms/bacterial_nomenclature.php). *Pseudomonas* species are ubiquitous in nature and can be isolated from humans, clinical samples, the plant rhizosphere, soil and water (including seawater) (Spiers *et al.*, 2000; Yamamoto *et al.*, 2000; Moore *et al.*, 2006).

In the present study, a polyphasic approach was undertaken to determine the taxonomic status of nine isolates recovered from an evaporative lagoon in the

Churince system, a hydrological system in the Cuatro Ciénegas valley in Coahuila, Mexico (26° 50.830' N 102° 09.335' W).

A large collection of bacterial isolates was obtained from surface water samples collected at two different points (10 m apart) in the lagoon referred to as Laguna Grande. Water samples (15 ml) from each site were collected in sterile BD Falcon vials (BD Biosciences) and 200 µl from each sample was inoculated on glutamate/starch/phenol red (GSP) agar plates (Kielwein, 1971). The GSP medium had the following composition (g l⁻¹): sodium L-(+)-glutamate, 10.0; starch (soluble), 20.0; potassium dihydrogen phosphate, 2.0; magnesium sulfate, 0.5; phenol red, 0.36; agar–agar, 12.0). Incubation was performed at 29 °C for 48 h. Colonies grown on different GSP plates were purified by subculture on Luria–Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) plates and the cultures were maintained at –80 °C in LB broth (1% tryptone, 0.5% yeast extract and 1% NaCl) with 20% (w/v) glycerol prior to analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of strains 1N^T and 3N are EU791281 and EU791282, respectively.

A figure showing a pulsed-field electrophoresis gel of novel strains described in this work and type and reference strains of known *Pseudomonas* species is available with the online version of this paper.

Isolates were grown in 5 ml LB broth. DNA was extracted by using a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Repetitive-sequence-based PCR profiles of the isolates and reference strains were determined with BOX primers and conditions as described by Versalovic *et al.* (1991, 1994). The PCR conditions consisted of an initial denaturing step at 95 °C for 7 min, 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 65 °C for 8 min and a final extension at 65 °C for 8 min. The PCR products were electrophoresed in 1.5 % agarose gels (0.5 × TAE buffer; 2.42 g Tris-HCl, 0.57 ml glacial acetic acid, 1 ml 0.5 M EDTA (pH 8.0) and 1 l distilled and deionized water) for 5 h at 180 mV (5 V cm⁻¹). The gels were stained with ethidium bromide and a digital image was obtained for further analysis. Isolates that displayed the same number of bands and similar fragment sizes were considered identical and therefore belonging to the same bacterial group or genotype. On this basis, nine isolates recovered from different water samples were identified as having identical (or very similar) repetitive-sequence-based PCR patterns (data not shown). 16S rRNA gene sequences were obtained from two of these strains, namely 1N^T and 3N, in order to define their taxonomic affiliation. The almost-complete 16S rRNA gene sequences were obtained by PCR amplification using primers 27F and 1492R and the conditions described previously (Lane, 1991). 16S rRNA gene sequences (approx. 1450 bp) were obtained (High-Throughput Sequencing Enterprise of the University of Washington, USA) and were compared with all publicly available complete 16S rRNA gene sequences of the type strains of *Pseudomonas* species with validly published names, using the Ribosomal Database Project (RDP) II database and software (Cole *et al.*, 2007). An

analysis based on 16S rRNA gene sequence similarities indicated that the closest relatives of strain 1N^T were *Pseudomonas peli* R-20805^T (98.5 %), *Pseudomonas argentinensis* CH01^T (97.5 %), *Pseudomonas flavescens* B62^T (97.4 %) and *Pseudomonas anguilliseptica* NCIMB 1949^T (97.4 %). Recently, two novel *Pseudomonas* species, *Pseudomonas guineae* and *Pseudomonas marincola*, were described (Bozal *et al.*, 2007; Romanenko *et al.*, 2008) that showed 97.9 and 97.6 % identity, respectively, with respect to strain 1N^T. The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) illustrates the position of strains 1N^T and 3N relative to members of *Pseudomonas* species; both strains clustered within a single group that was clearly separated from the cluster formed by the closely related type strains of the species *P. peli*, *P. anguilliseptica* and *P. guineae*, and from the clusters formed by the type strains of *Pseudomonas borbori*, *P. marincola*, *Pseudomonas segetis*, *P. argentinensis* and *P. flavescens*. These data suggested that the nine isolates analysed in the present study might constitute a novel *Pseudomonas* species.

The preparation of whole-cell proteins from all of the isolates and SDS-PAGE assays were performed as described previously (Estrada-De Los Santos *et al.*, 2001). The *Pseudomonas* isolates recovered from the lagoon shared almost-identical protein profiles, but their protein patterns were notably different from those of *P. anguilliseptica* LMG 21629^T and *P. peli* LMG 23201^T, as well as from those of representatives of other closely related *Pseudomonas* species (Fig. 2). It has previously been noted that bacteria with identical or similar protein patterns possess high levels of genome similarity (Vandamme *et al.*, 1996). On this basis, the SDS-PAGE results strongly suggested that all of the isolates represented a novel *Pseudomonas* species.

Fig. 1. Phylogenetic tree, based on Kimura two-parameter distances and neighbour-joining clustering after multiple alignment (1480 bp) of the 16S rRNA gene sequences of strains 1N^T and 3N and the most closely related members of the genus *Pseudomonas*. Bootstrap percentages (based on 1000 replications) >50 % are shown at branch points. The phylogenetic analysis was performed by using the software package MEGA, version 3.1 (Kumar *et al.*, 2004). The unweighted pair group method with arithmetic averages and the maximum-parsimony method were also used and gave approximately the same results, with the same clustering of the novel strains. Bar, 0.002 substitutions per site.

Fig. 2. Whole-cell protein profiles of strains 1N^T, 11N, 10N and 9N and of type strains of known *Pseudomonas* species. (a) Lanes: 1, *P. anguilliseptica* LMG 21629^T; 2, *P. peli* LMG 23201^T; 3–6, strains 1N^T, 11N, 10N and 9N, respectively; 7, *P. stutzeri* LMG 11199^T; 8, *P. flavescens* LMG 18387^T. (b) Lanes: 1–6, strains 1N^T, 3N, 2N, 9N, 8N and 6N, respectively; 7, *P. peli* LMG 23201^T.

For qualitative and quantitative analysis of the cellular fatty acids, strains 1N^T and 3N were cultivated on tryptic soy agar (Sigma-Aldrich) for 2 days at 28 °C. The whole-cell fatty acid compositions were determined by using an Agilent 6850 gas chromatograph (Agilent Technologies) and the fatty acid methyl esters were analysed by using the Sherlock 4.5 Microbial Identification System (Microbial ID). The fatty acid compositions of strains 1N^T and 3N, together with type-strain data for their closest phylogenetic relatives, are shown

in Table 1. The major fatty acid composition supports the affiliation of strains 1N^T and 3N to the genus *Pseudomonas*; in addition, there are differences in the relative abundance of fatty acids with respect to the type strains of *P. anguilliseptica*, *P. flavescens*, *P. guineae* and *P. peli*. Strains 1N^T and 3N exhibit clear quantitative differences for C_{12:0}, C_{12:0} 3-OH and C_{16:0} with respect to the type strains of their closest relatives, i.e. *P. anguilliseptica*, *P. marincola*, *P. guineae* and *P. peli*. In addition, these strains exhibit summed feature a (C_{16:1}ω7 and/or C_{16:1}ω6c), which is not present in the closely related species (Table 1). Although most of the fatty acids reported previously for the most closely related *Pseudomonas* species are present in the profiles of strains 1N^T and 3N, the absence of an unknown fatty acid (equivalent chain length 11.799) and the fatty acid components of summed feature b (C_{16:1}ω7 and/or iso-C_{15:0} 2-OH) is noteworthy. Special caution should be exercised with regard to comparison of the fatty acid profiles of 1N^T and 3N with that of *P. guineae* M8^T, as the latter was grown for 4 days at 15 °C before the fatty acid analysis, and this is not typical of the standard temperature ranges used for this type of analysis in members of the genus *Pseudomonas*.

To clarify the taxonomic relationships at species level, DNA–DNA hybridization experiments were performed as described previously (Estrada-De Los Santos *et al.*, 2001); the analysis was based on relative levels of hybridization to ³²P-labelled DNA from strain 1N^T. DNA–DNA relatedness assays were performed with four strains recovered from the lagoon, with the type strain of the most closely related *Pseudomonas* species (as indicated by 16S rRNA gene sequence data) and with the type strain of *Pseudomonas stutzeri*, which has a fatty acid profile very similar to those of 1N^T and 3N according to the database of the Microbial

Table 1. Cellular fatty acid compositions of strains 1N^T and 3N and strains of the most closely related *Pseudomonas* species

Taxa: 1, strain 1N^T; 2, strain 3N; 3, *P. peli* (LMG 23201^T and R-20815); 4, *P. anguilliseptica* LMG 21629^T; 5, *P. guineae* (three replicate readings of M8^T); 6, *P. marincola* JCM 14761^T; 7, *P. flavescens* LMG 18387^T. Data for *P. peli*, *P. anguilliseptica* and *P. flavescens* were taken from Vanparys *et al.* (2006), values for *P. guineae* were taken from Bozal *et al.* (2007) and values for *P. marincola* were taken from Romanenko *et al.* (2008). Results for groups of strains are expressed as means (with SD in parentheses). tr, Trace (<0.1%); –, not detected.

Fatty acid	1	2	3	4	5	6	7
C _{10:0} 3-OH	3.11	3.17	4.10 (0.23)	3.17	3.47 (0.58)	5.7	5.23
Unknown (ECL 11.799)*	–	–	4.01 (0.06)	3.14	3.70 (0.39)	1.2	tr
C _{12:0}	8.08	8.08	4.92	3.87	3.99 (0.49)	13.2	11.7
C _{12:0} 3-OH	3.69	3.67	4.17 (0.28)	2.7	3.61 (0.28)	3.7	4.85
C _{16:0}	14.85	15.16	10.01 (0.04)	23.17	10.92 (3.08)	20.2	19.15
C _{16:1}	–	–	–	–	–	18.6	–
C _{17:1} ω8c	tr	tr	3.17 (0.04)	tr	tr	3.7	tr
C _{18:0}	1.03	1.00	tr	tr	tr	–	tr
C _{18:1} ω7c	31.29	31.46	26.53 (0.61)	33.57	31.02 (1.08)	32.1	33.82
Summed features†							
a	34.79	34.70	–	–	–	–	22.07
b	–	–	27.55	26.81	37.81 (1.33)	–	–

*ECL, Equivalent chain length.

†Summed features: a, C_{16:1}ω7c and/or C_{16:1}ω6c; b, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Table 2. Enzyme activity and assimilation of carbon sources

Taxa: 1, 1N^T; 2, 3N; 3, *P. peli* LMG 23201^T; 4, *P. guineae* M8^T; 5, *P. anguilliseptica* LMG 21629^T; 6, *P. marincola* JCM 14761^T; 7, *P. flavescens* LMG 1837^T. All strains were positive for oxidase and catalase activity and for assimilation of malate. All strains were negative for the following: indole formation, fermentative acid production from glucose, urease, hydrolysis of aesculin and gelatin, β -galactosidase, *N*-acetylglucosamine and assimilation of D-ribose, maltose, adipate, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, amygdalin, arbutin, aesculin, ferric citrate, salicin, cellobiose, D-lactose (bovine origin), melibiose, D-sucrose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol and 5-ketogluconate. Data for *P. guineae* were taken from Bozal *et al.* (2007) and data for *P. marincola* were taken from Romanenko *et al.* (2008). NA, Data not available; w, weakly positive; +, positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Fluorescent pigments	–	–	–	–	–	–	+
Enzyme activity:							
Nitrate reduction	–	–	–	+	–	+	–
Arginine dihydrolase	–	–	–	–	–	+	–
Assimilation of:							
D-Glucose	–	–	–	–	–	–	+
Trehalose	–	–	–	–	–	–	+
L-Arabinose	–	–	–	–	–	–	+
D-Mannose	–	–	–	–	–	–	+
D-Mannitol	–	–	–	–	–	–	+
Caprate	+	+	–	+	+	+	+
Citrate (trisodic)	+	+	–	+	+	+	–
Glycerol	–	–	–	–	–	–	+
D-Xylose	+	+	–	–	–	–	–
D-Galactose	–	–	–	+	–	–	+
D-Fructose	–	–	–	–	–	NA	w
D-Mannose	–	–	–	–	–	–	+
L-Rhamnose	+	+	–	–	–	–	–
D-Mannitol	–	–	–	–	–	–	+
D-Fucose	+	+	–	–	–	–	–
Potassium gluconate	+	+	–	–	–	–	+
Potassium 2-ketogluconate	–	+	–	–	–	–	–
Phenyl acetate	–	–	–	–	–	+	–

Identification System (Microbial ID). The DNA–DNA reassociation values between the strains recovered from the lagoon were in the range 87–99 % (3N, 87 %; 2N, 96 %; 5N, 97 %), indicating close relationships at the species level (Vandamme *et al.*, 1996; Stackebrandt *et al.*, 2002). In contrast, low reassociation values (<15 %) were obtained in hybridizations of strain 1N^T with *P. peli* LMG 23201^T (14 %), *P. anguilliseptica* LMG 21629^T (14 %), *P. flavescens* LMG 18387^T (13 %) and *P. stutzeri* LMG 11199^T (12 %). These DNA–DNA reassociation data, together with the 16S rRNA gene sequence results, as well as the SDS-PAGE protein patterns and the fatty acid profiles, support the notion that the isolates recovered from the evaporating

lagoon represent a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas cuatrocienegasensis* sp. nov. is proposed.

To estimate the genome size of *P. cuatrocienegasensis* sp. nov., strains 1N^T, 2N, 3N, 6N, 8N, 9N, 10N and 11N were analysed by PFGE with *I-CeuI* endonuclease. Genomic DNA preparation and digestion of whole genomes were carried out as described by Liu *et al.* (1993) and Matushek *et al.* (1996), but with some modifications in the DNA-extraction procedure [3 h lysis time instead of overnight; two overnight washes of the lysed product instead of one; and eight washes with TE (10 mM Tris–Cl, pH 7.5; 1 mM EDTA) instead of two]. For *P. cuatrocienegasensis* sp. nov., the mean genome size was 2995 ± 663 kb, which is similar to those of *P. peli* LMG 23201^T (3450 kb) and *P. flavescens* LMG 18387^T (2665 kb), but much smaller than that of *P. aeruginosa* PAO1 (6262 kb). Four RNA operons were detected in *P. cuatrocienegasensis* sp. nov. strain 1N^T (Supplementary Fig. S1, available in IJSEM Online).

Bacteriological and biochemical characterization of the novel isolates was carried after growth in LB medium for 48–72 h at 29 °C. The production of fluorescent pigments was tested on King's B medium (King *et al.*, 1954). Other phenotypic features were assayed with the API 20NE and API 50CH systems according to the instructions of the manufacturer (bioMérieux). The API 20NE system was used to determine nitrate reduction, gelatin liquefaction, aesculin hydrolysis, urease activity, indole production, fermentative acid production from glucose, β -galactosidase activity and arginine dihydrolase activity. All colonies were grown on LB agar plates to determine the oxidase reaction as a complementary test in the API 20NE system. The utilization of various carbon sources was tested by using the API 50CH system after incubation for 72 h at 29 °C. The inoculation medium used for the API 50CH test strips was CHB/E [l^{–1}: ammonium sulfate (2 g), yeast extract (0.5 g), tryptone (1 g), disodium phosphate (3.22 g), monopotassium phosphate (0.12 g), trace elements (10 ml), phenol red (0.17 g); pH 7.4–7.8]; the incubation was performed at 20–25 °C. The results of these tests showed that the strains of *P. cuatrocienegasensis* sp. nov. differ from the most closely related species in terms of the utilization of specific carbon sources, e.g. D-xylose, D-fucose and gluconate; complete data are shown in Table 2.

Description of *Pseudomonas cuatrocienegasensis* sp. nov.

Pseudomonas cuatrocienegasensis (cu.a.tro.cie.ne.gas.en'sis. N.L. fem. adj. *cuatrocienegasensis* pertaining to the Cuatro Ciénegas valley, the location of the evaporative lagoon from which the organism was isolated).

Cells are rods (1.2 ± 0.13 µm long and 0.53 ± 0.02 µm wide) that are motile by means of a single, polar flagellum. Gram-negative, oxidase-positive and catalase-positive. Good growth occurs on LB agar and GSP agar at 28–32 °C; colonies on LB agar are beige, mucoid and irregular.

No production of fluorescent pigments is observed in King's B medium. The major fatty acids in the type strain are C_{16:0} (14.85 %), C_{18:1ω7c} (31.29 %) and summed feature a (C_{16:1ω7c} and/or C_{16:1ω6c}; 34.79 %); in addition, C_{12:0} (8.08 %), C_{12:0} 3-OH (3.69 %), C_{10:0} 3-OH (3.11 %), C_{18:0} (1.03 %) and C_{17:1ω8c} (<1 %) are detected. It is noteworthy that strains 1N^T and 3N lack an unknown fatty acid (equivalent chain length 11.799) that is present in type strains of all of the most closely related species. The carbon sources utilized are indicated in Table 2. The utilization of D-xylose, L-rhamnose, D-fucose and potassium gluconate serve as differential characteristics for this species.

The type strain, 1N^T (=LMG 24676^T=CIP 109853^T), was isolated from an evaporative lagoon in Cuatro Ciénegas, Coahuila State, Mexico.

Acknowledgements

We thank R. González-Chauvet (Biósfera-UNO, México), G. M. Rosas-Barrera (Instituto de Ecología, UNAM, México), A. Casamitjana and the Modern American School (México) for sample collection and technical assistance. We gratefully acknowledge Bulmaro Reyes-Coria (Instituto de Investigaciones Filológicas, UNAM, México), Jean Euzéby (Ecole Nationale Vétérinaire, Toulouse, France) and Bernhard Schink (Universität Konstanz, Germany) for help with the etymological construction of the novel species name, G. Delgado-Sapien (Facultad de Medicina, UNAM, México) for assistance with the molecular techniques, A. Patrón-Soberano (Instituto de Fisiología Celular, UNAM, México) for the micrographs and L. E. Eguiarte (Instituto de Ecología, UNAM, México) for comments and ideas. This project was supported by grants from SEMARNAT/CONACyT to V. S. (2002-CO1-0246) and by a CONACyT-UNAM scholarship to A. E. E.

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